LEUKODEPLETION FILTERS FOR PREVENTION OF TRANSFUSION TRANSMISSION OF LEISHMANIA

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1. ABSTRACT

BACKGROUND: Leishmania is an intracellular parasite of monocytes transmissible by transfusion. The feasibility of reducing Leishmania with leukodepletion filters was studied. At collection, infected blood contains the amastigote form of Leishmania within monocytes. Amastigotes cause the rupture of monocytes releasing free amastigotes that convert to promastigotes which exist extracellularly at blood storage temperatures. Leukodepletion filters were tested at various time points in this process.
STUDY DESIGN AND METHODS: Blood products were infected with Leishmania organisms then filtered: 1. using whole blood filters at collection, 2. using bedside filters after storage, and 3. to determine whether free promastigotes could be eliminated.
RESULTS: Filtration at collection reduced Leishmania by three to four logs or to the level of detection. Filtration of infected packed red cells after two weeks of storage showed a reduction of Leishmania by four logs. Filtration resulted in a six to eight log reduction in promastigotes in either the presence or in the absence of white cells within the filter.
CONCLUSION: Filtration at the time of collection and after storage of Leishmania infected blood resulted in a substantial reduction of free and intracellular organisms. There is currently no donor screen for Leishmania. Until adequate testing is developed, the use of leukodepletion filters could add to the safety of the blood supply.

2. INTRODUCTION

Leishmania infects 12 million people in 88 countries, with 600,000 new clinical cases reported annually and many more unreported. Leishmaniasis is responsible for 57,000 deaths annually (1). Leishmania infection is transmitted to humans via the bite of the phlebotomine sand fly which acts as a vector (2). Transmission of Leishmania by transfusion has also been reported (3, 4, 5, 6, 7, 8, 9, 10), and there are rare reports of transplacental and sexual transmission (11, 12). There is no blood donor screen for Leishmania. Leishmania is endemic in Iraq and Afghanistan where soldiers from nonendemic areas are currently deployed. Until adequate methods for screening can be implemented, ways to eliminate the risk of pathogens transmissible by transfusion are needed.

The life cycle of the Leishmania protozoa involves two stages. The promastigote is the form of the organism which exists in the gastrointestinal tract of the sand fly, and is the initial form injected into the skin of the human host. Rapidly after injection, these organisms are phagocytized by monocytes and transform into amastigotes. Amastigotes reproduce in macrophages, are released, and are rapidly taken up by monocytes in the blood and in the remainder of the reticuloendothelial system (2, 13, 14). At any given time the overwhelming majority of Leishmania organisms reside within reticuloendothelial cells and do not circulate freely in the blood of an infected human host. With blood donation, at the time of blood collection, organisms are present inside monocytes. Upon storage at 4^{o} C the organisms will remain within white cells for some time, but eventually emerge as free amastigotes which may transform into promastigotes surviving extracellularly in the stored blood (15).

Leukodepletion filters have been used to reduce transmission of organisms such as cytomegalovirus (CMV) which exist intracellularly within white blood cells (16, 17, 18, 19), and have been used to prevent transmission of the intracellular organisms HTLV-I (20), and the rickettsial species *Orientia tsutsugamushi* (21). Although *Trypanosoma cruzi* is not an intracellular organism, leukodepletion filters have been suggested as a means of reducing this organism in infected blood (22). Since the Leishmania genus of organisms are intracellular pathogens of monocytes, it seemed theoretically possible to use leukodepletion filters as a means of removing the organism from infected blood products at the time of collection when the organism is intracellular.

Leukodepletion filters of different types are designed to be used at the time of collection or at the time of transfusion. This research was initially undertaken to study the feasibility of eliminating or reducing the transfusion transmission of Leishmania using leukodepletion filters at the time of blood collection. It was proposed that if monocytes containing amastigotes were removed from the transfusion product at

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collection, the possibility of transmission of Leishmania by transfusion of blood from donors at risk would be reduced or eliminated. Since leukodepletion filters are not always used at collection, and some white cells will die and degrade during blood storage; the efficacy of bedside leukodepletion after blood storage was also tested. In a third phase of the study, filters were specifically evaluated for their ability to remove extracellular promastigotes which could eventually emerge during prolonged storage of blood. There is currently no donor screen for Leishmania, but leukodepletion filters are widely available and could be implemented immediately in at risk areas where they are not already in use.

3.MATERIALS AND METHODS

Maintenance of Leishmania Cultures
Leishmania promastigotes were cultured at 22^o C in Schneider's Drosophila medium (Invitrogen, Carlsbad, CA) supplemented with 30% heat inactivated fetal calf serum (Invitrogen). Cells were maintained in logarithmic phase by seeding at 2 x 10^6/mL. Stationary phase promastigotes were obtained when cultures approached 2 x 10^7/mL. Cell counts were made by counting cell numbers in a hemocytometer. One hundred million Leishmania were used to infect monocytic cells which were later added to blood products.

Evaluation of Leishmania Cultures
Cultures were examined every day for a period of 28 days using an inverted microscope with a 40 x objective (Olympus CKX41, Tokyo, Japan) . If there were any mobile promastigotes, the culture was considered positive. If none were detected after 28 days, the culture was considered negative.

Leishmania Organisms
Organisms used to infect blood products were isolated from soldiers seen at Walter Reed Army Medical Center and were characterized at Walter Reed Army Institute of Research Leishmania Diagnostic Laboratory by cellular acetate electrophoresis of isoenzymes (23).

Blood Collection
All blood collection occurred within an institutional review board approved protocol and informed consent was obtained. All blood collection and processing followed AABB and Food and Drug Administration guidelines.

I. Filtration at Collection
Monocyte Isolation and Culture with *Leishmania*
One hundred mL of freshly donated whole blood was collected from each of four healthy volunteer blood donors (different individuals from unit donors) and mononuclear cells were purified by density sedimentation using BD Vacutainer CPT Tubes containing Sodium citrate. Mononuclear cells were spun at 1500 x g for 45 minutes at 22^o C, washed twice in Hank's Balanced Salt Solution, and spun at 180 x g for 10 minutes at room temperature. The mononuclear cells were resuspended in 50 mL of RPMI FBS 1640 medium (Invitrogen), and were then cultured in 50 cc of RPMI FBS 1640 medium at 37^o C in 5% CO_2 overnight with one hundred million stationary phase Leishmania promastigotes suspended in 1 mL of RPMI FBS 1640 medium. Then the flasks were gently washed three times with RPMI FBS to remove extracellular promastigotes. (Incubation overnight at 37^o C leads to phagocytosis of promastigotes which transform into amastigotes intracellularly.) After washing, adherent monocytes were scraped from the flasks and spun into a pellet. The supernatant was removed, and the pellet was resuspended in 1 mL of RPMI FBS. The resuspended pellet was added to a freshly collected unit of blood product. This procedure was repeated for each blood product which was infected.

This process reliably produced over 90% infection of monocytes with an average of 4 amastigotes per infected monocyte as determined by light microscopy of Giemsa stained preparations. The presence of viable parasites in these monocytes was also determined by analysis of conversion of amastigote to promastigote from cultures of infected monocytes serially diluted in culture.

Whole Blood Collection
Four units of fresh whole blood were collected from volunteer blood donors into blood collection bags containing CPD. After infection of units with Leishmania, packed red cells were prepared by centrifugation of whole blood at 5000 x g with expression of plasma into a satellite bag. One hundred mL of AS-5 was added.

Infection of Whole Blood Units with Leishmania and Filtration of Whole Blood
Each of the resuspended pellets of Leishmania infected monocytes was added to one of the units of whole blood on the day of collection. The units were then divided into two equal aliquots using a sterile tubing welder (SCD 312 Terumo Corporation, Tokyo, Japan) to add a second bag. One aliquot was filtered (WBF2 Leukocyte Filter for Whole Blood, Pall, East Hills, NY) as fresh whole blood, and the other was left unfiltered. Filtered units were spun down into plasma and packed red cells. The packed red cells and plasma were cultured in ten fold dilutions to determine the lower limit of detection of organism. Half of an mL of packed red cells or plasma or 0.5 mL of each ten fold serial dilution was added to 10 mL of Schneider's Drosophila medium supplemented with 30% heat inactivated fetal calf serum and incubated at 22^o C. The units were stored, packed red cells at 4^o C and fresh frozen plasma at -20^o C. The units of packed red cells were cultured at weekly intervals for the storage life of the units. Plasma was frozen and cultured again upon thawing.

II. Bedside Filtration

Blood Collection

Four units of fresh whole blood from four volunteer donors were collected into CPDA-1 blood collection bags. Blood was centrifuged at 5000 x g and the plasma expressed into a satellite bag, producing packed red blood cells at an approximate hematocrit of 70%.

Infection of Packed Red Blood Cells with Leishmania and "Bedside" Filtration

Four aliquots of infected monocytes were prepared as stated above under "Monocyte Isolation and Culture with Leishmania", and used to infect four units of packed red blood cells on the day of collection . After addition of amastigote laden macrophages, packed cells were stored at 4^{o} C. After two weeks of storage, 0.5 mL of each of the four units of packed red cells were cultured in 10 fold serial dilutions to determine the limits of detection of infecting organism pre-filtration. The units were immediately filtered using RCXL1 High Efficiency Leukocyte Reduction Filter (Pall) for Bedside Red Cell Transfusion, and 0.5 mL of each of the filtered units was cultured in 10 fold serial dilutions.

III. Filtration of Promastigotes

Blood Collection

From each of three volunteer donors, 350 mL of plasma was collected by apheresis using a COBE Spectra Cell Separator (Gambro BCT, Lakewood, CO) and frozen at -20^{o}C for later use. From three additional volunteer donors, 450 mL of Group O fresh whole blood was collected in CPDA-1 blood collection bags.

Infection of Plasma with Leishmania Promastigotes and Filtration

For each of three replicates, 350 ml of apheresis plasma was thawed and divided into two unequal aliquots of 250 mL and 100 mL by using a sterile tubing welder for the addition of a second bag . One hundred mL of plasma was used to prime a WBF2 Leukocyte Filter for Whole Blood, and 450 mL of whole blood was used to prime a second filter. One hundred million free promastigotes of *Leishmania donovani infantum* were added to the bag containing the remaining 250 mL of plasma. The infected plasma was cultured in ten fold serial dilutions to determine the limits of detection. The 250 mL of infected plasma was divided into 2 equal aliquots of 125 mL each, using the sterile tubing welder for the addition of another bag. One aliquot was run through each of the primed filters. The filtered products were cultured in 10 fold serial dilutions to determine the ability of the filters containing white cells and without the presence of white cells, to remove free extracellular promastigotes from the infected plasma.

4. RESULTS

The number of infected monocytes added to each blood product is summarized in Table 1.

Table 1

Number of Infected Monocytes x 10^{6} Added to Each Blood Product (Monocytes averaged 4 amastigotes per cell.

	Filtration at:	
Unit	Collection	Bedside
1	28.4	14.0
2	7.98	29.5
3	19.05	8.65
4	12.7	24.6

I. Filtration of Whole Blood at Collection with Leukodepletion Filters Removes Leishmania

Removal of white cells at the time of collection was evaluated as a means of removing Leishmania from infected units of blood, because at the time of blood collection, organisms are present inside monocytes. Each of four units of whole blood infected with Leishmania was divided into two parts, one filtered and one unfiltered. Units were cultured at weekly intervals beginning on the day of collection. Depending on the time of sampling, organisms were detectable in 1 to 4 log ten dilutions of packed red cells (Figure 1), but in only 1 to 2 log ten dilutions of plasma (Figure 2). Filtration of blood at collection, with subsequent separation into packed red cells and plasma showed a significant reduction of Leishmania by up to three to four logs or to beyond the level of detection.

Of the four units tested, unit 1 was infected with *Leishmania major* and units 2, 3, and 4 were infected with *Leishmania donovani infantum*. *L. donovani* survived up to 42 days and *L. major* 28 days (Figure 1). It should be noted that survival of Leishmania in stored packed red cells was longer than what was seen in a previous publication (13); however, in that study the organism was *L. tropica* with a survival in packed red cells of only 25 days which is similar to what was seen in our study with *L. major*.

As in previous work (13) none of our organisms survived freezing in plasma, but recovery of organism in cultures of fresh plasma did occur for *L. donovani*. Not unexpectedly, the growth of the organism in plasma was less than its growth in red cells since in the separation of red cells from plasma; the buffy coat containing infected white cells was left predominantly in the red cell fraction.

II. Bedside Filtration of Packed Red Blood Cells with Leukodepletion Filters Removes Leishmania

At two weeks of storage, 4 units of Leishmania infected packed red blood cells were filtered using bedside leukodepletion filters, and were cultured just prior to and just after filtration. Unfiltered units showed growth of organism in culture in up to 3 log dilutions of the units while filtered units showed no growth of organism. This demonstrates an efficacy to the level of detection which is a 4 log reduction. Growth of organism is shown prefiltration and post filtration for each of four units of packed red cell units infected with *Leishmania donovani infantum* amastigote laden macrophages using a bedside red cell leukodepletion filter after two weeks of storage at 4^{o} C (Figure 3).

III. Leukodepletion Filters Result in the Removal of Extracellular Leishmania Promastigotes

When free Leishmania promastigotes were added to plasma, whole blood filters were effective in removing free promastigotes from the infected plasma (Figure 4). Promastigotes were detectable in 5 to 7 log dilutions of plasma with a reduction by filtration of 6 to 8 logs.

The efficacy of the filters in removing promastigotes was theorized to be due to three potential mechanisms which include the uptake of organisms by the trapped white cells within the filter, by mechanical trapping, or by charge related interactions between the organism and the filter fibers. In an attempt to determine what mechanism is involved in the removal of free organisms, the promastigotes were passed through filters primed with fresh frozen plasma alone, which would not contain white cells, or through filters primed with the white cells from a full unit of fresh whole blood.

The filters were equally effective in removing the organisms with or without white cells contained within the filters. This suggests that the mechanism of efficacy of the filters to remove promastigotes is not entirely dependent upon the presence of white blood cells, and is more likely to be due to mechanical trapping or charge related factors. It would appear that the filters were more effective in removing promastigotes than they were in removing white cells containing amastigotes. Amastigotes in white cells showed a 3 to 4 log reduction (Figures 4 and 5), because of the 3 to 4 log limitation of the filter in leukocyte removal, while promastigotes showed a 6 to 8 log reduction (Figure 4).

5. DISCUSSION

We have evaluated the use of leukodepletion filters for reduction of Leishmania in three different situations: at the time of collection, after storage, and in the removal of extracellular organisms. Since Leishmania are intracellular within white blood cells at the time of collection of infected blood products, it was initially expected that the use of filters would only be effective when used at that time. There was a three to four log reduction seen with filtration of whole blood at collection. This is consistent with the claims of the filter manufacturers of a 3 to 4 log reduction of white blood cells by leukodepletion filters. At the time of collection when the organism is contained within monocytes, the removal of organisms is dependent upon the ability of the filter to remove leukocytes.

This effective reduction occurred in spite of the large number of organisms with which these units were infected. In this experiment a far greater load of organism was used than would be expected in a unit collected from an asymptomatic blood donor(24). At any given time the overwhelming majority of Leishmania organisms reside within reticuloendothelial cells and do not circulate freely in the blood of an infected human host. Leishmania circulates intermittently and at low density in healthy individuals (24). The 3 to 4 log limits of white cell removal by the filter is consistent with the growth of organism in filtered unit number 3 cultured on day14 and unit 4 cultured on day 0 when the parasite load is over 4 logs, since the leukodepletion filters tested are only able to remove 3 to 4 logs of white cells. This does not represent a failure of the filter; it shows that the filters when used to remove infected leukocytes have a 3 to 4 log limit of reduction.

At some time points demonstration of reduction by filtration was somewhat dependent on level of detection. When the level of organism was low, it was only possible to show reduction to the level of detection. This does not necessarily indicate that this is the maximal efficacy of the filter. When the level of white cells containing Leishmania was high, there were times when the filter was only able to reduce the level of organism by three logs, but for free organism that was not the case. The filters were more effective in removing free organism than in the removal of leukocytes.

It should be noted that in the filtration of whole blood only half units were filtered. It is possible that a different result could be obtained if whole units were filtered, but this is unlikely since the magnitude of reduction was in the order of several logs of ten vs. the filtration of half a unit versus a full unit.

Since universal leukoreduction at the time of collection is not performed in all areas of the world, the efficacy of bedside filters to remove Leishmania from stored packed red cells was also assessed. Week two was the time when the greatest number of organisms was present in unfiltered units (Figure 1). At two weeks of red cell storage some white cells survive, while some have degraded and ruptured releasing free organism. This time point seemed to present the greatest challenge to the bedside filter in that the number or organisms would be greatest, and in that there would be free organisms in addition to organisms contained within leukocytes. After two weeks of storage of packed red cells, leukodepletion filters still showed a four log reduction in the growth of Leishmania. At two weeks of storage, free Leishmania is present in addition to Leishmania within leukocytes. The success of filters in the removal of Leishmania at two weeks, suggests that filters are capable of removing free organism in addition to removing leukocytes containing Leishmania.

The success of filters in removing free promastigotes is not entirely unprecedented, since leukodepletion filters have been shown to be somewhat effective in the removal of the trypomastigote form of *Trypanosoma cruzi*, which has a similar size, shape, and surface charge to the promastigotes of Leishmania species (22). The efficacy of filters to remove Leishmania after storage brought up the question of what the mechanism might be which allowed the removal of Leishmania from the blood when it is extracellular.

A 3 to 4 log reduction at the time of collection is consistent with removal of organism within white cells since the manufacturer claims a 3 to 4 log reduction of leukocytes. Additional mechanisms of efficacy are also suggested by the differing level of reduction, 6 to 8 logs, seen in the experiments in which free promastigotes were filtered. It has been hypothesized that this may be occurring by mechanical trapping, by charge related interactions, or by interactions with white blood cells also trapped within the filters.

In an attempt to answer part of the question of mechanism, an experiment was designed in which free extracellular promastigotes were added to plasma. The infected plasma was divided into identical aliquots, and was filtered in two different ways. In one set of experiments the filters were primed with plasma alone, and in the other experiments the filters were primed with a unit of fresh whole blood which would contain many white cells that become trapped inside the filter. The infected plasma was then filtered using both types of primed filters. The presence of white blood cells within the filters did not change the level of removal of organism, and the free promastigotes were removed equally by filters primed in both ways. In fact the removal of promastigotes was more effective than the removal of white cells in that a 6 to 8 log reduction was seen. This suggests that the mechanism of efficacy of the filters in removing promastigotes is not entirely dependent upon the presence of white blood cells and may also be related to charge or mechanical trapping of promastigotes.

Leishmania promastigotes and amastigotes are known to have a negative surface charge (25, 26, 27, 28, 29). Charge related interactions between white cells and filter fibers has been proposed as a possible mechanism for the removal of white cells by the filter. It is entirely plausible that this is also the mechanism for removal of free extracellular Leishmania organisms. This possibility should be investigated, since it might be applicable to the development of filters designed to remove other pathogens.

This study is small, but based on this work, we can conclude that until adequate donor testing for Leishmania is developed, the use of leukodepletion filters at the time of collection or at the bedside could add to the safety of the blood supply by reducing the number of organisms contained in an infected unit of blood. In endemic areas where large portions of the population are infected, the use of filters would reduce the risk especially in infants and immuosuppressed individuals. Leukodepletion filters are in use in some, but not all endemic areas. Prevalence needs to be determined among donors who reside in or have traveled to endemic areas such as deployed troops and contractors sent to Iraq and Afghanistan. There is no data to indicate that the current one year donor deferral is adequate.

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Figure 1 Leukodepletion Filters Used at Collection Reduce Leishmania in Packed Red Blood Cells Half of each of four units of Leishmania-infected whole blood was filtered at collection and separated into packed red blood cells and plasma. Packed red blood cells were stored at 4o C for 42 days and were sampled at 7 day intervals beginning on the day of collection. Filtered and unfiltered units were cultured in 10 fold dilutions to determine the level of detection of organism. The number of log ten dilutions of growth of organism in filtered and unfiltered units is compared. This graphically represents the number of logs of reduction in growth of organism by filtration. The 3 to 4 log limits of white cell removal by the filter is consistent with the growth of organism in filtered units number 3 and 4 when the parasite load is over 4 logs. This is consistent with a white cell reduction by the filters of up to 4 logs when used at the time of collection while the organism is intracellular.

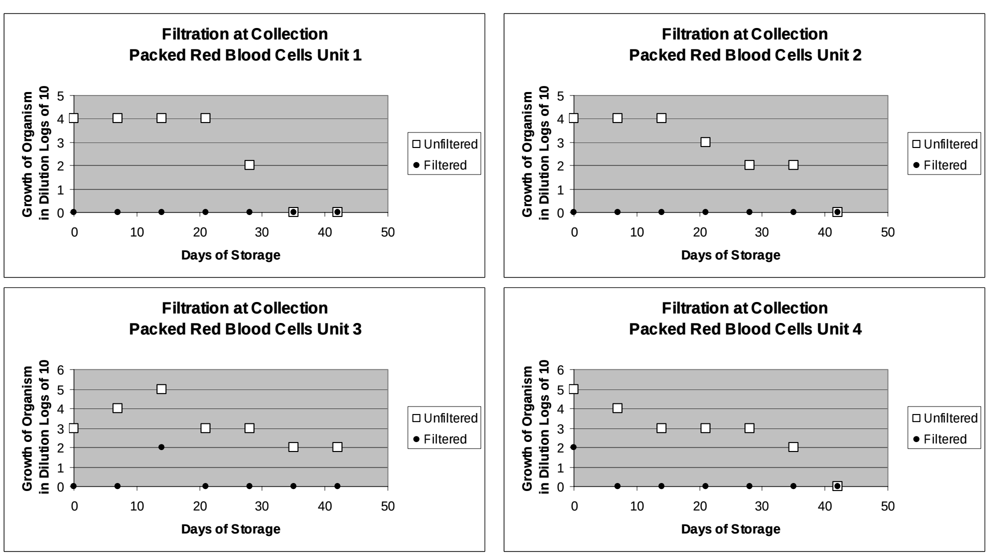

Figure 2 Leukodepletion Filters Used at Collection Reduce Leishmania in Plasma Half of each of four units of whole blood was filtered at collection and separated into packed red blood cells and plasma. Plasma was cultured in 10 fold dilutions to determine the level of detection of organism pre and post freezing at -80o C. No growth of Leishmania was seen in unfiltered or filtered units after freezing. The number of log ten dilutions of growth of organism in filtered and unfiltered units is compared. This graphically represents the number of logs of reduction in growth of organism by filtration. Demonstration of reduction by filtration is limited by the presence of a low burden of organism in unfiltered plasma since most of the leukocytes remain with the packed red blood cells during plasma preparation.

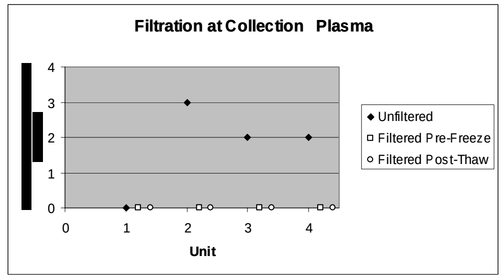

Figure 3 Bedside Leukodepletion Filters Reduce Leishmania in Stored Packed Red Blood Cells Four units of Leishmania infected packed red blood cells were stored for 2 weeks at 4o C, and units were cultured before and after filtration. Each filtered or unfiltered sample was cultured in 10 fold serial dilutions to determine the level of detection of organism. The number of log ten dilutions of growth of organism in filtered and unfiltered units is compared. This graphically represents the number of logs of reduction in growth of organism by filtration.

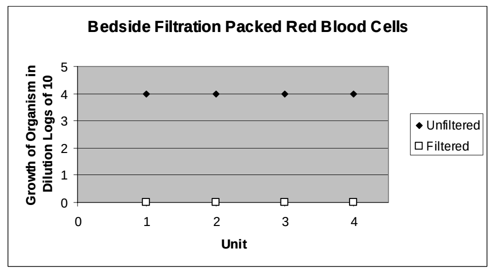

Figure 4 Leukoreduction Filters Remove Free Leishmania Three units of fresh apheresis plasma were infected with Leishmania promastigotes and each was divided into 2 aliquots, one filtered and one filtered with a filter primed with leukocytes. Each sample was cultured in 10 fold serial dilutions before and after filtration to determine the level of detection of organism. The number of log ten dilutions of growth of organism in filtered and unfiltered units is compared. This graphically represents the number of logs of reduction in growth of organism by filtration. The presence of leukocytes within the filter was not necessary for the reduction to occur by filtration and did not alter the reduction of organism.